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Rosmarinic Acid Effect on Bonding Performance to Caries Affected Dentine

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Abstract

Aim of the study: This study investigated the effect of applying rosmarinic acid (RA) as dentine surface pretreatment on the immediate microtensile bond strength (µTBS) after 24h of water storage and delayed µTBS after 5000 thermocycles using 1-step self-etch adhesive (1-SEa) on artificial caries affected dentine (ACAD).

Material and method: In this experimental *in vitro* study, the dentine surface of 40 premolars were exposed and subjected to a pH-cycling procedure for ACAD induction. Next, teeth were divided randomly into two groups (n=20 each): ROS-group that was treated with RA solution (100µM RA in 5% ethanol) for 5s then dried for 5s, and control untreated group. One sample was taken from each group for surface analysis with SEM. Following bonding and composite build up procedures, the specimens were sectioned into 1mm^2 beams and subjected to microtensile test after 24h (immediate) or 5000 thermal cycles (delayed). The fractured beams were examined under stereomicroscope to determine failure type. Data were analysed with t-test of independent variables, significance level was set at 0.05.

Results: ROS-group showed a significant increase in the immediate µTBS compared to control (p=0.003), while the µTBS of both groups were decreased significantly following thermocycling (p<0.001), without any difference between them (p=0.1598).

Conclusion: Rosmarinic acid can enhance only the immediate bond strength of 1-SEa on ACAD.

Keywords: Caries affected dentine, Cross-linkers, Rosmarinic acid, Polyphenols, 1-step self-etch adhesive, bond strength.

Introduction

Dental adhesives depend on the creation of the hybrid layer when adhering dentine (Nakabayashi et al., 1982; Meerbeek et al., 1992). The stability and structural integrity of collagen fibrils and polymeric chains inside the hybrid layer are essential for durable bonding (Nakabayashi et al., 1998; De Munck et al., 2005). Despite the development in dental adhesives, the enzymatic degradation of the collagen matrix has been observed as a pathway for bond degradation, which reduces the lifespan of the bonded interface (Breschi et al., 2008). Therefore, techniques that boost the ability of collagen to withstand degradation are anticipated to enhance the durability of dentine bonding.

Recently, collagen cross-linkers have been shown to limit bond degradation. This is achieved by slowing matrix metalloproteinase enzyme (MMP) biodegradation and strengthening type-I collagen fibrils to resistant to biodegradation (Seseogullari-Dirihan et al., 2016; Mazzoni et

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al., 2017). The tensile features of collagen fibrils can be improved by the intrinsic chemical cross-links between the side chains of amino acids within collagen molecules (Goh et al., 2007). To improve the fibrillar resistance against enzymatic degradation and to be raise the tensile characteristics of the dentine matrix by the production of extra inter- and intramolecular cross-links, it has been proposed that dentine matrices might be biomodified through the process of extrinsic collagen cross-linking. (Bedran-Russo et al., 2008; Fang et al., 2012; Hass et al., 2016).

Natural polyphenols have been found to enhance the process of collagen cross-linking. Polyphenolic substances are distinguished by the presence of at least one aromatic ring within their structure, together with one or more hydroxyl groups. The presence of hydroxyl groups in polyphenols is responsible for their antioxidant capacity. Moreover, the structures that are rich in hydroxyl groups enable them to form complexes with proteins, particularly proline-rich proteins found in dental collagen (Bravo, 1998). The reinforced crosslinking interactions improves the mechanical strength of dental bonding by increasing its resistance to external forces (Yang et al., 2017; Yi et al., 2019; Peng et al., 2020). The polyphenolic chemicals have the ability to bind to metal ions and outcompete peptidases, such as MMP, for collagen's catalytic domain (Mazzoni et al., 2018). Consequently, the breakdown of collagen in the hybrid layer by enzymes would be hindered, thereby preserving the durability of the bond between the adhesive and dentine (Epasinghe et al., 2012; Yang et al., 2016; Yang et al., 2017).

Rosmarinic acid (RA) is one of the natural antioxidants that derived from the plant rosemary and belongs to polyphenols. It possesses cross-linking and MMP inhibitory properties in addition to its antioxidant effect (Aruoma and Cuppet, 1997; Cadenas and Parker, 2001; Radziejewska et al., 2018). In previous studies, it was shown that using a rosmarinic acid solution as a pretreatment alone or following sodium hypochlorite treatment can maintain bond stability between 2-step self-etch adhesive (2-SEa) on both normal and CAD after one year of storage in artificial saliva (Prasansuttiporn et al., 2017; Prasansuttiporn et al., 2020).

Also, the effect of RA on bond stability of total etch adhesive to normal dentine has been investigated (Ruksaphon and Pisol, 2017). However, there is a limitation of data on the impact of RA on the bonding capacity of 1-SEa on CAD.

The aim of this study was to investigate whether the application of RA as pretreatment could enhance the immediate and delayed µTBS of the simplified 1-SEa when CAD is the bonding substrate. The null hypothesis was as follows: applying RA would not enhance the immediate and delayed bond strength of 1-SEa to artificial CAD.

Methodology:

Teeth selection and preparations:

This experimental *in vitro* study included 40 sound human premolars that were extracted for orthodontic purposes. The age of patients ranged from (18-25) years old (Awad and Al-Zaka., 2023). The minimum sample size was determined based on data from a previous study (Lemos et al., 2022). The alpha level (α) was determined to be 0.05, the statistical power (1-β) to be 0.80 and large effect size. The study was conducted after obtaining ethical authorization from the institutional ethics committee board of the College of Dentistry, Mustansiriyah University (NO: MUOPR29). Teeth were kept for 48 h in 0.1% thymol, then washed thoroughly and stored in distilled water until used (Salah and Sleibi, 2023). For acrylic blocks construction, the roots were impeded to a level of 2mm below the cemento-enamel junction inside a silicone mould filled with cold cure acrylic (Acrosun, Tehran, Iran) (Mohammed R. 2010). The flat dentine surface of teeth was exposed by occlusal cusp elimination with a cutting disc (Komet-Brasseler, Lemgo, Germany) under running water. Next, a #600-grit silicon paper was used under copious water for 30 s to polish dentine surface and standardise the smear layer (Nicoloso et al., 2016).

pH- cycling protocol for artificial CAD:

Before conducting the pH-cycling procedure, the dentine surface was left exposed while the remaining tooth parts were coated with two coats of acid-resistant varnish. Each specimen was then socked in 10 ml of fresh demineralization solution for 8 hours. For the following 16 hours, the specimens were soaked in the same volume of freshly prepared reminralization solution (Nicoloso et al., 2016). The procedure was done for 14-day period. Teeth underwent a thorough rinsing process with deionized water (Albayati, Baghdad, Iraq) for 1 min to eliminate any residual traces of the immersing solutions. Finally, the specimens were preserved in deionized water (Al-Obaidi and Jasim, 2023).

Preparation of RA solution:

The RA used in this study was provided as a powder with a purity of at least 98% (Cayman, MI, USA). The necessary quantity was measured using a high-precision five-digit analytical balance (Kern, Balingen, Germany). The powder was then dissolved directly in a 5% ethanol aqueous solution. The final solution contained 100µM of RA in a 5% ethanol solution (pH=5.4) (Prasansuttiporn et al., 2020). In order to make the 5% ethanol solution, concentrated ethanol (Honeywell Riedel-de Haën, Seelze, Germany) was first diluted with distilled water. A solution of rosmarinic acid was made and utilised on the day of the experiment following the manufacturer's recommendations (Prasansuttiporn et al., 2011).

Dentine surface treatment and bonding-restorative procedure:

Teeth were randomly divided into two groups n=20 each,

I: ROS-group: the dentine surface was treated with a single droplet of RA solution that equally disseminated across the entire surface with a regular-sized microbrush with gentle

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strokes, for a duration of only 5 seconds. Subsequently dried for 5 seconds. (Figure 1)

II: Control group: dentine surface was irrigated with water and air dried.

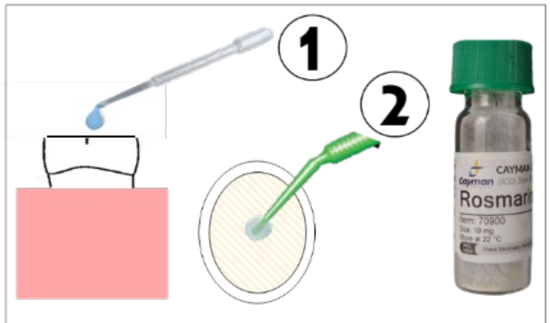

Figure 1: RA application 1: RA applied with plastic dropper. 2: gently distributed all over the dentine surface microbrush.

Next, universal adhesive Clearfil S^3 Bond Universal Quick (Kuraray, Tokyo, Japan) was applied with a regular-sized microbrush in self-etch mode in a single layer, and then gently air dried for ≥ 5 s until the adhesive did not move. This is in accordance with the recommendations made by the manufacturers. With zero waiting time, adhesive was photo-cured for 10 s using then photo-cured with the multimode light-emitting diode light-curing device (1000 mW/cm2 & 385–515 nm, Eighteeth, Changzhou, China). Composite was constructed to 4mm height in two 2mm increments using Clearfil AP-X (Kuraray, Tokyo, Japan) and photoactivated for 20 s using a light-curing device (1500 mW/cm2 & 385-515 nm, Eighteeth, Changzhou, China). The power of the curing device was checked periodically using a portable radiometer (Woodpecker, Guangxi, China). (Table 1) lists the study's dental materials. Following composite build up, specimens were stored for 24h at 37°C in distilled water (Prasansuttiporn et al., 2011).

Table 1: Some of the materials used in this study.

Materials (Manufacture)	**Composition by the manufacture**	**pH**
Clearfil AP-X (Shade A2)	Bisphenol adiglycidyl methacrylate, triethylene glycol dimethacrylate, silanated barium glass filler, silanated silica filler, silanated colloidal silica, camphorquinone, initiators, accelerators, pigments	N/A*
Clearfil S^3 Bond Universal Quick	10-methacryloyloxydecyl dihydrogen phosphate, bisphenol adiglycidyl methacrylate, ethanol, 2-hydroxyethyl methacrylate, hydrophilic amide monomer, colloidal silica, silane coupling agent, sodium fluoride, camphorquinone, water.	2.3

*N/A: not applicable

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Microtensile bond strength test and thermocycling:

After 24h of water storage, a low-speed automated diamond saw (Isomet 4000, Buehler, Lake Bluff, USA) was used to slice the specimens perpendicular to the adhesive interfac in bucco-lingual and mesio-distal directions to obtain composite-dentine beams (surface area: 1.0×1.0 mm). In order to release the beams, the last horizontal cuts were made at the cemento-enamel junction level. The measurements of the beams were determined using a digital calliper (Total tools, Selangor, Malaysia) (figure 2). Two beams were obtained from each tooth (20 teeth x 2 beams=40 beams per group). Half of the beams (n=20/group) were subjected to immediate µTBS testing, whereas the remaining beams were subjected to simulated artificial ageing through 5000 thermal cycles (SD mechatronik, Westerham, Germany) within a temperature range between 5ºC and 55ºC, with a dwell time of 30 s and a transfer time of 5 s (Shioya et al., 2021). Next, the beams were individually adhered at both ends using cyanoacrylate glue (Akfix 705 fast adhesive, Istanbul, Turkey) to the attachment jig with a minimum distance of 1mm from the adhesive interface. Subsequently, the jig was fixed into a universal testing machine (Instron, MA, USA) (figure 3). Then subjected to tensile force at a cross-head speed of 0.5mm/min until bonding failure of the specimen occurred (Armstrong et al., 2017). Bond strength was calculated in Megapascal (MPa) (Bluehill Lite software, Instron, MA, USA).

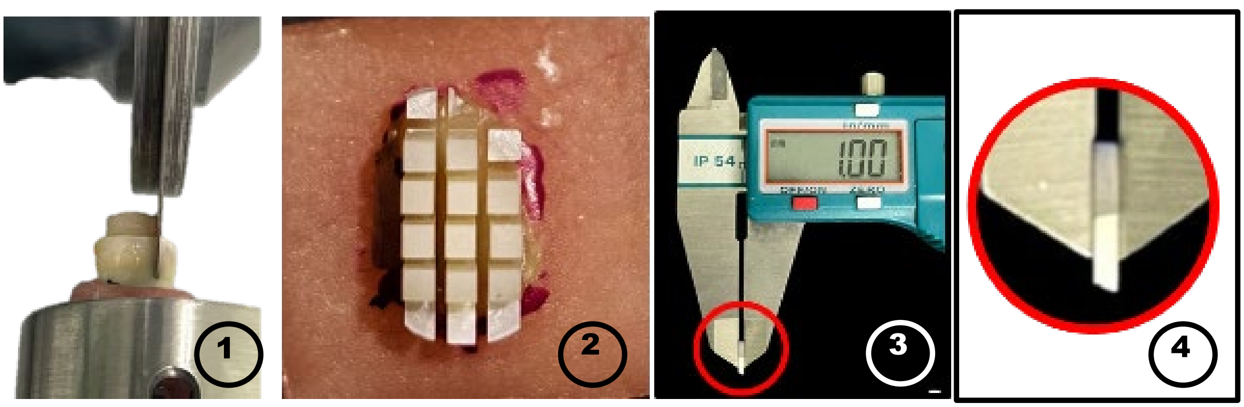

Figure 2: Beams preparation 1: teeth sectioned by Isomet device. 2: direction of sections was in bucco-lingual and mesiodistal directions perpendicular to the adhesive interface 3,4: beam diameter checked with digital calliper.

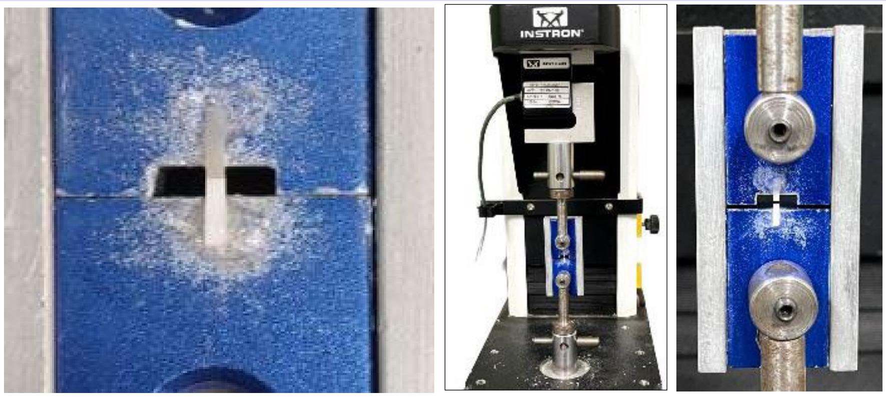

Figure 3: Microtensile procedure

Failure mode:

For the purpose of determining the mode of failure, the fractured beams were inspected by one trained examiner at two different times (intra-examiner) using a stereomicroscope (MA 100 Nikon, Tokyo, Japan) at 40x magnification. The failure was categorised according to Nicoloso et al., 2016 as either adhesive, occurring at the contact between the resin and dentine, cohesive failure, occurring completely inside the dentine or resin composite, or mixed failure, involving both adhesive failure at the interface and cohesive failure of the adjacent substrate (Nicoloso et al., 2016).

Preparation of samples before scanning electron microscopy (SEM):

Scanning electron photomicrographs were taken to evaluate changes in dentine surface morphology after pH-cycling and RA treatment. For the preservation of collagen network and improving image quality preparation steps consist of fixation, dehydration and drying with hexamethyldisalazine were done. Teeth were soaked in 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7 for 12 hours at 4ºC. The teeth were then cleaned with 0.2M phosphate buffer for 1 h and deionized water for 1 min (Zuluaga-Morales et al., 2022) Subsequently, the specimens were dehydrated using increasing concentrations of ethanol: 25% for 20 min, 50% for 20 min, 75% for 20 min, 95% for 30 min, and 100% for 60 min. Afterwards, the specimens immersed in hexamethyldisalazine (Aldrich chemical co, Milwaukee, USA) for 10 minutes for dehydration (Shioya et al., 2021). Finally, the sides of the dentine were sprayed with gold, and then they were examined at under microscope at 10000x by two examiners (inter-examiner).

Statistics:

The SPSS Statistics 26.0 software (IBM, Chicago, USA) was used to do the statistical tests. The beam was used as a statistical unit for the μTBS data. The Shapiro-Walk test shows that all data was normal distributed ($p>0.05$) and the comparisons among groups were analysed with t-test of independent variables. Failure mode distributions were analysed using the chi-square test. The threshold of significance has been set to 0.05.

Results:

Results of μTBS:

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Table 2 shows the mean values of the microtensile bond strength, standard deviation comparisons among groups. At 24h, ROS-group showed significantly higher bond strength compared to control (p=0.003). Following thermocycling, the µTBS of ROS and control groups were comparable ((p=0.1598). Furthermore, the results showed a significant decrease in the µTBS of both groups after thermocycling in comparison with the results of 24h (p<0.001).

Table 2: µTBS means (±SD) in MPa of the study groups (n=10 teeth per subgroup, 20 beams per sub group)

Time	Groups	Means ± (SD)	*P* value
24h	ROS	25.31± (4.4)	p=0.003 S
	Control	20.70± (4.8)	
TC	ROS	10.18± (2.1)	p=0.1598 NS
	Control	8.91± (3.3)	
Groups	**Time**	**Means ± (SD)**	***P* value**
ROS	24h	25.31± (4.4)	p<0.001 S
	TC	10.18± (2.1)	
Control	24h	20.70± (4.8)	p<0.001 S
	TC	8.91± (3.3)	

t-test of independent variables shows the significance between groups, the significance level was *p<0.05).*
SD= standard deviation
n=number of samples
MPa= Megapascal
S=significant
NS= non-significant

Results of failure mode:

Mixed and adhesive failure were observed figure 4. The adhesive failure is the predominant type in both groups. The results of chi-square test show no correlation between failure type and the groups p=0.189 neither between time and groups at different times (Table 3). The failure mode distribution in all groups were summarised in Figure 5.

Table 3: Failure mode analysis with Chi-Square test.

Correlation between failure type and groups.					
Group	**Failure type**		**X^2**	**P value**	**df**
	Adhesive	**Mixed**			
ROS (n=40)	28 (70%)	12 (30%)	1.72	0.189NS	1

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Control (n=40)	33 (82.5%)	7 (17.5%)			
Total=80	61 (76.25%)	19 (23.75%)			
Correlation between failure type and groups at 24h.					
Group/ at 24h	Failure type		X^2	P value	df
	Adhesive	Mixed			
ROS (n=20)	10 (50%)	10 (50%)	1.66	0.1967NS	1
Control (n=20)	14 (70%)	6 (30%)			
Total=40	24 (60%)	16 (40%)			
Correlation between failure type and groups after TC.					
Group/TC	Failure type		X^2	P value	df
	Adhesive	Mixed			
ROS (n=20)	18 (90%)	2 (10%)	0.36	0.5483NS	1
Control (n=20)	19 (95%)	1 (5%)			
Total=40	37 (92.5%)	3 (7.5%)			

X^2=Chi-Square value
df=degree of freedom
NS= non-significant.

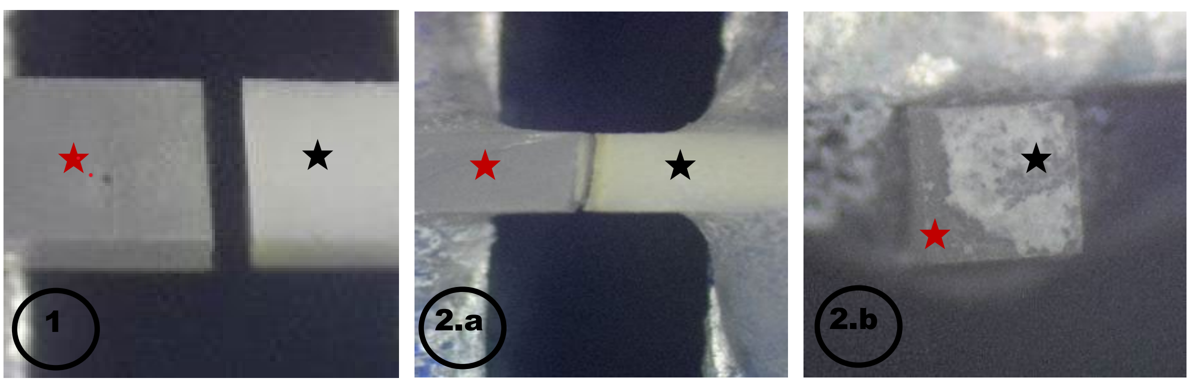

Figure 4: Fractured beams under stereomicroscope. 1: adhesive failure between dentine and resin composite, 2: mixed failure, a. side view of the fractured beam, b. top view of the fractured beam showing composite surface with retained pieces of dentine. (The black star refers to dentine while the red one refers to composite)

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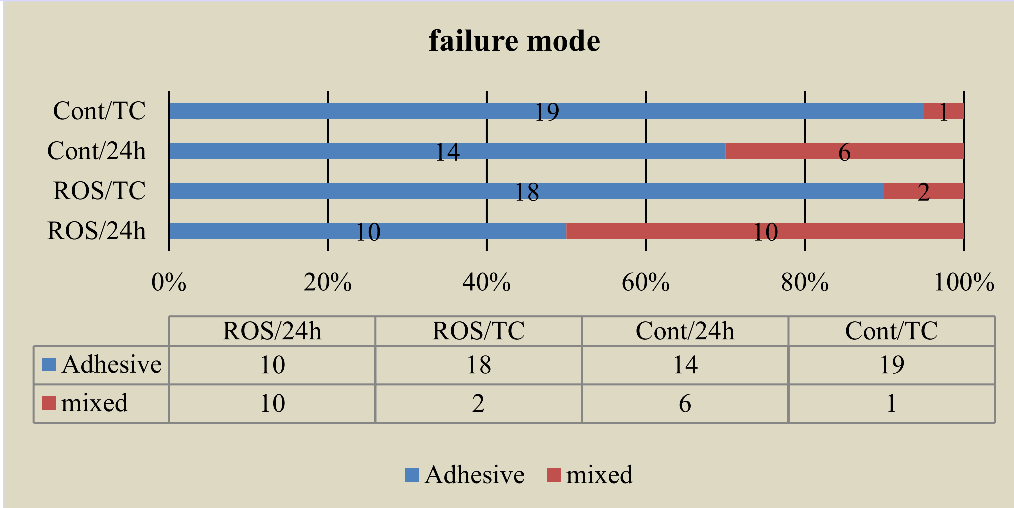
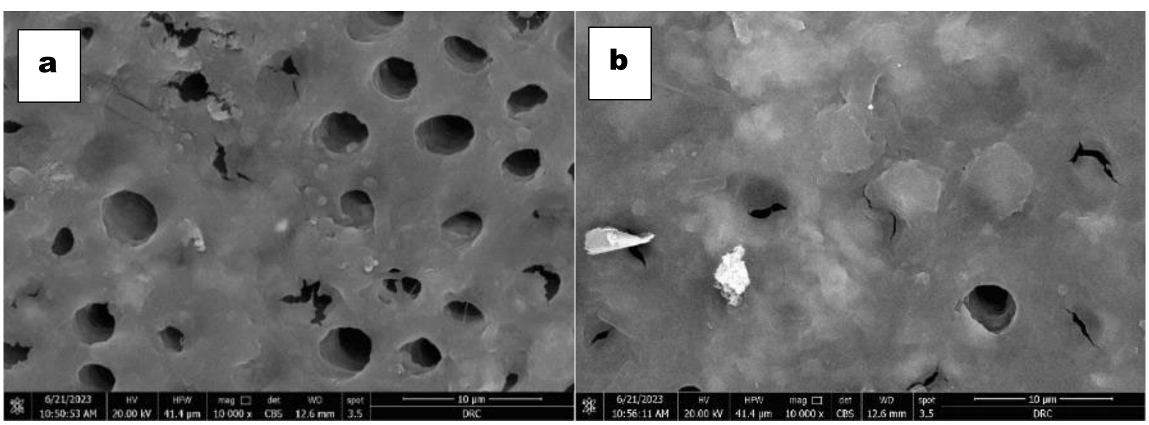


Figure 5: Number of fractured beams in each subgroup (n= 20 beam)

Results of SEM:

The surface of ACAD showed an exposure of the dentinal tubules related to the effect of pH-cycling while some of dentinal tubules were covered with smear figure (5.a, b). The surface morphology of tooth sample treated with rosmarinic acid showed opened and large diameter dentinal tubules and some of orifices shows funnelling effect figure (5.c).



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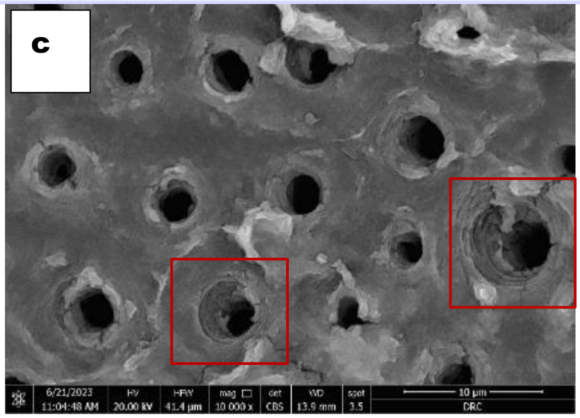

Figure 5: SEM analysis: a &b) dentine surface of ACAD after pH-cycling procedure. c: dentine surface following RA treatment. Red box showing the funnel shape of the orifices

Discussion:

The present study intended to investigate the influence of applying rosmarinic acid on the effectiveness of 1-step self-etch Clearfil Tri S Bond Universal Quick adhesive that was applied to artificially caries-affected dentine. The findings demonstrated a statistically significant improvement in the immediate bond strength subsequent to rosmarinic acid application in ROS-group as compared to the control group. The stability of the bond strength upon artificial ageing with 5000 thermocycles was not sustained, as accelerated deterioration in the bond strength occurred. Accordingly, the null hypothesis regarding the effect on the immediate bond strength was rejected and accepted regarding delayed bond strength.

In the clinical scenario, the substrate being bonded often is CAD rather than normal dentine; dental adhesives exhibit reduced effectiveness on CAD compared to normal dentine, therefore, the long-term stability of resin restorations may be significantly compromised (Isolana et al., 2018; Follak et al., 2021). The caries process in CAD can cause morphological, chemical, and physiological changes that may hinder the penetration of adhesive monomers into the underlying dentine. This can lead to the formation of a defective hybrid layer (Wang et al., 2007), which in turn increases the risk of hydrolytic bond degradation over time (Nakajima et al., 2005; Arrais et al., 2004). Consequently, numerous recent investigations have concentrated on improving adhesion to CAD (Taniguchi et al., 2009; Kunawarote et al., 2011; Prasansuttiporn et al., 2020; Follak et al., 2021).

In this study, the application of RA solution as a primer resulted in a significant increase in the immediate µTBS in ROS-group compared to the control group. This could be attributed to the cross-linking ability of RA. Rosmarinic acid, also known as a-o-caffeoyl-3,4-dihydroxyphenyllactic acid, is a chemical consisting of two catechol rings (1,2-dyhydroxy-benzene) (Aruoma and Cuppet, 1997; Lau et al., 2015) (figure 6). These rings are responsible for the polarity of rosmarinic acid. The antioxidant activity of rosmarinic acid is due to the capacity of catechol to

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establish an intermolecular hydrogen link between the available hydrogen of its hydroxyl and phenoxyl radicals, thereby enhancing the stability of its radicals. In addition to its antioxidant activity, rosmarinic acid also exhibits a cross-linking effect and MMP-inhibitory impact (Aruoma and Cuppet, 1997; Cadenas and Parker, 2001; Radziejewska et al., 2018).

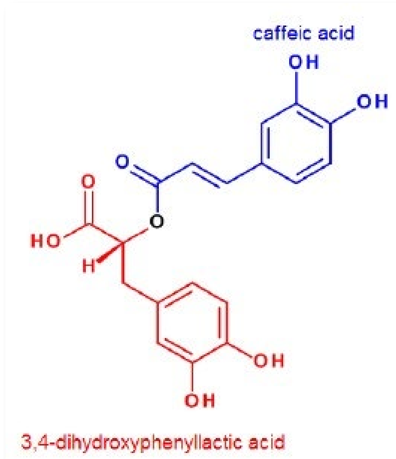

Figure 6: The chemical structure of rosmarinic acid (Lau et al., 2015).

The interactions between RA and proline-rich proteins, such as type-I collagen in dentine, are responsible for its cross-linking effect. Thereby increasing the mechanical properties of collagen fibres and subsequently improving adhesion to dentine (Zhao et al., 2022). Surface analysis by SEM showed widely open dentinal tubules with funnel orifices compared to control that may indicate surface demineralization; however, the pH of RA solution was 5.4 and the application time was 5s only; this appeared to be insufficient to cause surface demineralization. Further investigations about this speculation are needed. From the failure mode, even there was no significance between the groups. It's worth mentioning that the mixed type was more in ROS group; this could be due to the cross-linking effect in enhancing the mechanical properties of collagen fibres, while the adhesive type, which is the worst type, was mostly in the untreated control group.

For the delayed bond strength, both groups exhibited a notable decline in bond strength following 5000 thermal cycles. Ruksaphon reported that applying rosmarinic acid to normal dentine prior to adhesion with total etch adhesives could maintain the stability of the bond strength for up to 3 months of aging, while the bond strength significantly decreased at 6 months of water storage as a higher nanoleakage was expressed at the resin/dentine interface (Ruksaphon and Pisol, 2017). This was disagreed with in previous studies by Prasansuttiporn, who revealed that RA, when used prior to 2-SEa on both normal and caries affected dentine, showed no improvement in the immediate bond strength, while it was able to maintain bond stability over 1 year of storage in artificial saliva (Prasansuttiporn et al., 2017; Prasansuttiporn et al., 2020). This disagrees with our study.
According to the last systematic review (Zhao et al., 2022), polyphenol compounds are able to increase the immediate bond strength, although RA was not included in this

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systematic review since there are still limited studies available. However, the similarity in the chemical structure among polyphenol compounds makes them share the same properties. The cross-linking effect may be affected by different factors such as the dentine properties, type of adhesive, concentration and application time of the cross-linker (Pinto et al., 2015; Carli et al., 2018), so further studies are required to include other types of adhesives taking into consideration the other factors.

Durable bonding to CAD remains a challenge, even with the advancement in adhesives (Follak et al., 2021). The efficacy of self-etching adhesives (SEa) affected by the properties of CAD. SEa is dependent on the chemical interaction with calcium ions, which are typically present in CAD at low concentrations (Isolana et al., 2018). In addition, the low acidity of these adhesives limiting their ability to demineralize and generate microporosities in CAD. Furthermore, due to the high porosity of CAD, SEa has been reported to form a thick and porous hybrid layer that plays a crucial role in increasing bond degradation between interfaces over time (Nakajima et al., 2011).

Another point worth mentioning is the adhesive used in this study, which based on the "no wait concept" technology, as it is possible to apply and directly photocure with no waiting time. Several recent in vitro investigations showed conflicting findings when they compared the "no-waiting" concept to a 10-s application approach (Kuno et al., 2019; Sato et al., 2018; Saito et al., 2020; Huang et al., 2017; Tugba et al., 2022). According to Saikaew et al., the reduction in application time could be associated with the presence of pores in the adhesive interface. These pores resulted from the incomplete evaporation of solvent and water (Saikaew et al., 2016). This could explain the high percentage of adhesive failures in the present study. The residual solvent and moisture could have a diluting effect and consequently reducing the bonding efficacy. Other factors that could have an impact on the bond strength include the utilisation of thermal cycles in artificial aging (Morresi et al., 2014; Morresi et al., 2015). Despite being a commonly employed approach for ageing, there is a noticeable absence of a universally accepted and standardised strategy for thermal cycling (Morresi et al., 2014). Additionally, the preparation of specimens with 1mm^2 beams would put up the effect of thermocycling since each 1 mm^2 beam were completely exposed from all their outer surfaces compared to conventional tensile strength with a larger sample size.

In the present study, using naturally affected dentine was challenging. This is because of the complexities associated with acquiring standardised substrates with such numbers of included samples that have CAD. Thus, artificially affected dentine was employed, which appeared to be one of the study limitations as it is challenging to fully replicate the chemical and histologic properties of natural CAD. Another limitation was using only one type of adhesive.

Conclusion:

Rosmarinic acid improves the immediate bond strength of 1-step self-etch adhesive, while it shows no advantage in maintaining the longevity of bond strength over 5000 thermal cycles.

Supplementary Material

None.

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Data Availability Statement

Data are available from the authors upon reasonable request.

Conflict of interest

The authors reported that they have no conflicts of interest.

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